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Fungicidal effect of photodynamic therapy against fluconazole-resistant Candida albicans and Candida glabrata

Fungicidal effect of photodynamic therapy against fluconazole-resistant *Candida albicans* and *Candida glabrata*

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Summary

Although photodynamic therapy (PDT) has shown great promise for the inactivation of *Candida* species, its effectiveness against azole-resistant pathogens remains poorly documented. This *in vitro* study describes the association of Photogem® (Photogem, Moscow, Russia) with LED (light emitting diode) light for the photoinactivation of fluconazole-resistant (FR) and American Type Culture Collection (ATCC) strains of *Candida albicans* and *Candida glabrata*. Suspensions of each *Candida* strain were treated with five Photogem® concentrations and exposed to four LED light fluences (14, 24, 34 or 50 min of illumination). After incubation (48 h at 37 °C), colonies were counted (CFU ml^{-1}). Single-species biofilms were generated on cellulose membrane filters, treated with 25.0 mg l^{-1} of Photogem® and illuminated at 37.5 J cm^{-2}. The biofilms were then disrupted and the viable yeast cells present were determined. Planktonic suspensions of FR strains were effectively killed after PDT. It was observed that the fungicidal effect of PDT was strain-dependent. Significant decreases in biofilm viability were observed for three strains of *C. albicans* and for two strains of *C. glabrata*. The results of this investigation demonstrated that although PDT was effective against *Candida* species, fluconazole-resistant strains showed reduced sensitivity to PDT. Moreover, single-species biofilms were less susceptible to PDT than their planktonic counterparts.

Key words: Photodynamic therapy, *Candida albicans*, *Candida glabrata*, fluconazole-resistance, light emitting diode.

Introduction

Oral candidiasis is a common opportunistic infection of the oral cavity caused by *Candida* species, the commonest being *Candida albicans*.[1,2] Recently, infections with species other than *C. albicans*, notably *C. glabrata*, have been increasingly described.[3] Numerous predisposing factors for oral candidiasis have been recognised.[1,2,4,5] Alterations in immune status associated with the AIDS epidemic, cancer chemotherapy, and organ and bone marrow transplantation has been often related to the increase in the incidence of *Candida* infections.[1,4] In addition, the use of dental prosthesis and subsequent biofilm formation on epithelial surfaces and prosthetic devices is critical in the development of denture-associated candidiasis, which is a frequent condition occurring in denture wearers.[5] Considering the high frequency of *Candida* infections in immunocompromised patients, it is clear that effective antifungal therapy is necessary. Topical antifungal agents are often prescribed to manage oral candidiasis.[6,7] However, these agents achieve only a transient response and relapses are frequent.[8,9] As the recurrence rate is high, systemic azole antifungals (e.g. fluconazole, itraconazole) have been largely used for the treatment of fungal infections.[8,10,11] Nevertheless, the increased use of azoles, combined with several cases of treatment failures, has drawn attention to the problem of antifungal drug resistance.[12–14]

Clinical resistance to antifungal drugs is a broad concept describing failure of an antifungal therapy, which results in persistence or progression of an infection.[14,15] An organism that is resistant to a drug prior to exposure is described as having primary or intrinsic resistance. Secondary resistance develops in response to exposure to an antimicrobial agent over long periods.[14] It has been already demonstrated that exposure to fluconazole provided resistance in the *C. albicans* population of HIV-positive patients[13] and also led to the replacement of fluconazole-susceptible *C. albicans* strains with other species that are intrinsically less fluconazole sensitive, such as *C. glabrata* and *C. krusei*.[13,14] The fungistatic activity of the azoles has also been associated with the failure of antifungal therapy in immunocompromised patients. For these agents, host defences are important contributors to the cure of the infection.[16,17] Another aspect related to antifungal resistance and recurrence of infection is the ability of *Candida* spp. to form biofilms on surfaces.[14,18] A biofilm has been defined as a community of microorganisms organised at interfaces, enclosed in a self-produced polymeric matrix and adhered to an inert or living tissue.[19] The presence of an exopolymeric matrix couple with the organisation of layers of cells may confer protection on organisms in the inner layers contributing to antifungal resistance.[18]

To overcome the problems associated with antifungal resistance, photodynamic therapy (PDT) has been evaluated as a promising method of treatment of oral candidiasis.[20–23] PDT involves the use of a photosensitising compound and a light source.[22,24] After the target cells were treated with the photosensitiser (PS), irradiation with non-thermal visible light of a suitable wavelength (the maximum absorption of the PS), in the presence of oxygen, would excite PS to initiate chemical reactions. The production of free radicals and other reactive oxygen species, such as singlet oxygen, leads to cellular damage, membrane lyses and protein inactivation.[24,25] Notably, the mechanism of PDT inactivation of fungi is completely different from that of antifungal agents. The reactive oxygen species promote perforation of the cell wall and membrane, thereby permitting the PS to translocate into the cell. Once inside the cell, oxidising species generated by light excitation induces photodamage to internal cell organelles and cell death.[25,26]

Although a number of studies have shown the susceptibility of *Candida* species to PDT,[20,21,26,27] there are still some aspects that remain to be better elucidated *in vitro*. Because of the non-specific oxidising agents, organisms resistant to conventional antifungal agents could be successfully killed by PDT and the development of resistance to such therapy (secondary resistance) seems to be unlikely. It has been already shown that repeated photosensitisation does not induce resistance in microorganisms.[28] Nevertheless, some studies have demonstrated that drug-resistant strains are less vulnerable to PDT than drug-susceptible organisms.[17,29] There are only a few reports available regarding the susceptibility of fluconazole-resistant *C. albicans* strains to PDT.[17,23] Considering that the most promising advantage of PDT would be the treatment of infections resistant to antifungal agents, the susceptibility of resistant *Candida* species to PDT should be better documented. The purpose of this study was to evaluate the efficacy of PDT in killing fluconazole-resistant (FR) strains of *C. albicans* and *C. glabrata*. The investigation was designed to find the minimal PS concentration and light fluence for the complete inactivation of the strains in the planktonic mode. Then, the most promising combination of PS and light were evaluated against single-species biofilms of *C. albicans* and *C. glabrata*.

Materials and methods

Photosensitiser and light source

Photogem® (Limited Liability Company Photogem, Moscow, Russia) was used as PS as it has regulatory approval for clinical use and it has been largely used in cancer phototherapy. This PS is a haematoporphyrin derivative (HpD) produced in Russia, very similar to Photofrin II. The two PSs present similar molecular structure as well as the ratio between monomer and oligomers in the lyophilised powder form.[30] Stock solutions of Photogem® were prepared by dissolving the powder in sterile saline and kept in the dark until use. Photogem® was excited by an LED (light emitting diode) light in the blue region of the spectrum, which PS absorbs more efficiently than red light (Fig. 1).

A LED device, named Bio Table, was designed by Instituto de Física de São Carlos (University of São Paulo, São Carlos, SP, Brazil). This system is composed of eight royal blue LEDs (LXHL-PR09, Luxeon® III Emitter; Lumileds Lighting, San Jose, CA, USA) uniformly distributed throughout the device. The LED device covered the wavelength range from 440 to 460 nm, with maximum emission at 455 nm. The intensity of light delivered was 12.5 mW cm^{-2}. The fluences tested were 10.5; 18.0; 25.5 or 37.5 J cm^{-2}. To achieve those fluences, the exposure time was calculated with the dosimetry formula: Fluence (J cm^{-2}) = Intensity of light (W cm^{-2}) × exposure time (s).
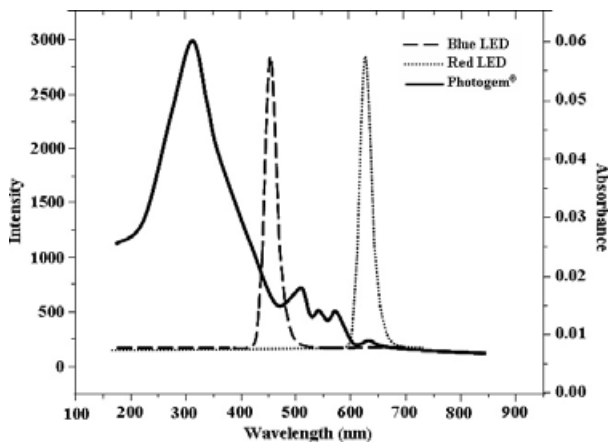
Figure 1 Emission spectrum of the blue and red LED (light emitting diode) light and the absorbance spectrum of Photogem® at concentration of 50 mg l^{-1}.

Fungal strains

Six FR clinical patient isolates were used in this study (*C. albicans*: 10R, 15R, 23R; *C. glabrata*: 50R, 63R, 87R). In addition, American Type Culture Collection strains (ATCC; Rockville, MD, USA) of *C. albicans* (ATCC 90028) and *C. glabrata* (ATCC 2001) were included as reference strains (Table 1). The minimum inhibitory concentration (MIC) for each strain was determined using the broth microdilution reference method[31] for fluconazole sensitivity. Isolates were maintained in solid yeast-peptone-glucose medium and frozen at −70 °C.

Photodynamic treatment against planktonic cultures

The yeast were individually inoculated in 5 ml of tryptic soy broth (TSB) and grown aerobically overnight at 37 °C. Each culture was centrifuged at 2000 rpm for 10 min, and the pellet washed twice with sterile distilled water and resuspended in sterile saline to a turbidity 10^6 cells ml^{-1} (McFarland standard). Aliquots of 100 µl of each *Candida* standardised suspension were

individually transferred to separate wells of a 96-well microtitre plates. An equal volume of PS solutions was added to each well to give final concentrations of 2.5, 5.0, 10.0, 25.0 and 50.0 mg l^{-1}. After incubation in the dark for 30 min (pre-irradiation time), each plate was placed on the LED device. Illumination was performed for 14, 24, 34 or 50 min, resulting in a total fluence of 10.5, 18.0, 25.5 or 37.5 J cm^{-2} respectively (P+L+). To determine whether PS alone had any effect on cell viability, additional wells containing the yeast suspensions were exposed to PS under identical conditions to those described above, but not to LED light (P+L−). The effect of LED light alone was determined by exposing cells to light without being previously exposed to PS (P−L+). Suspensions exposed to neither PS nor LED light acted as overall control (P−L−). To determine the cell survival, aliquots of the contents of each well were serially diluted 10-fold in sterile saline. Triplicate 25 µl aliquots were spread over the surfaces of Sabouraud dextrose agar (SDA) plates containing 5 mg l^{-1} gentamicin. All the plates were aerobically incubated at 37 °C for 48 h.

Based on the experiments described above, the most effective PS concentration and light fluence were selected and their long-term fungicidal effect against the yeast suspensions was determined. The experimental protocol was carried out as outlined above except that aliquots of the yeast suspensions were transferred to TSB tubes immediately after PDT procedures and incubated at 37 °C for further 7 days. Cultures were interpreted as positive or negative growth.

Photodynamic treatment against *Candida* biofilms

Yeast isolates were individually incubated overnight at 37 °C in TSB and diluted in fresh medium to a turbidity 10^6 cells ml^{-1} (McFarland standard). Aliquots of the standardised cultures were transferred to the surface of cellulose nitrate membrane filters (0.2 µm pore size, 13 mm diameter – Sartorius Stedim Biotech S.A, Germany) to generate single-species biofilms. Briefly, the membranes were placed on the surface of SDA, inoculated with the standardised cultures and incubated aerobically for 48 h at 37 °C. Following incubation, the membrane filters were removed aseptically from the agar plate and transferred slowly, so as to avoid any disruption of the biofilm, into separate wells of a 24-well microtitre plates containing 500 µl of Photogem® solution at 25 mg l^{-1}. After incubation in the dark (30 min; pre-irradiation time), the plates were placed on the LED device and illuminated for 50 min (37.5 J cm^{-2}). Control membranes were exposed to

Table 1 Original source of clinical isolates and ATCC strains.

Strains identification	Species	Original source
10R	*Candida albicans*	Esophagus
15R	*C. albicans*	Oropharynx
23R	*C. albicans*	Oral infection
ATCC 90028	*C. albicans*	Blood
50R	*Candida glabrata*	Urine
63R	*C. glabrata*	Urine
87R	*C. glabrata*	Urine
ATCC 2001	*C. glabrata*	Faeces

neither PS nor LED light. The membrane filters were then carefully transferred into 5 ml of sterile saline and vortexed for 1 min to resuspend the cells from the biofilms. Ten-fold serial dilutions were generated from the fungal suspensions and plated on SDA in triplicate. The plates were then aerobically incubated at 37 °C for 4 days.

After incubation, yeast colony counts of each plate were quantified using a digital colony counter (CP 600 Plus, Phoenix Ind Com Equipamentos Científicos Ltda, Araraquara, SP, Brazil). The colony forming unit per millilitre (CFU ml^{-1}) was determined.

Statistical analysis

Each experimental treatment with the PS concentrations in the presence of the mentioned light fluences was repeated three times. For the purposes of analysis, CFU ml^{-1} values were transformed into logarithm values (log$_{10}$). For the planktonic culture results, comparisons between two logarithms were performed using the unpaired Student's *t*-test. The results of PDT against biofilms were evaluated using the analysis of variance (ANOVA) and Tukey Honestly Significant Difference (HSD) *post hoc* test. Differences were considered statistically significant at $P < 0.05$.

Results

The results of MIC studies on *Candida* strains confirmed that the clinical isolates of *C. albicans* and *C. glabrata* were resistant to fluconazole, while the ATCC strains were susceptible to this drug. The MIC values found for *C. albicans* strains were >64, 16, >64 and 1 µg ml^{-1} for 10R, 15R, 23R and ATCC 90028 respectively. For *C. glabrata* strains, the MIC values established were >64, 16, 64^1 and <8 µg ml^{-1} for 50R, 63R, 87R and ATCC 2001 respectively.

According to the results obtained with planktonic cultures, the association of specific Photogem® concentrations and blue LED light fluences resulted in inacti-

vation of FR and ATCC strains. The inactivation (complete killing) was accepted when no evidence of growth on plates was observed after 48 h at 37 °C. Complete killing of the three FR *C. albicans* strains was achieved with 50.0 mg l^{-1} of PS associated with illumination at 18.0 J cm^{-2}. Inactivation of FR *C. albicans* with lower PS concentrations was verified by increasing the light fluence to 25.5 and 37.5 J cm^{-2} (Fig. 2a). In comparison with the ATCC strain (Fig. 2b), FR *C. albicans* required higher PS concentrations to inactivate it at 25.5 and 37.5 J cm^{-2} ($P < 0.05$). The mean CFU ml^{-1} values obtained from PDT against the three FR *C. glabrata* are illustrated in Fig. 3a. Complete killing of the FR strains of this species was observed with 25.0 mg l^{-1} of PS, after illumination at both 25.5 and 37.5 J cm^{-2}. At fluence of 37.5 J cm^{-2}, *C. glabrata* ATCC was killed with a lower PS concentration than that required for FR strains (Fig. 3b). In summary, the association of 25.0 mg l^{-1} with 37.5 J cm^{-2} resulted in complete inactivation of all microorganisms, irrespective of the species and strain evaluated.

The inactivation of each fluconazole-resistant strain was also evaluated separately. It can be seen from Table 2 that the PS concentration required for photoinactivation of each of the *C. albicans* FR strains was different at 18.0 and 25.5 J cm^{-2}, while the PS concentration for killing each of the *C. glabrata* FR strains differed only at 18.0 J cm^{-2}. However, high light fluences led to a homogeneous pattern of photoinactivation among the three FR strains of the same species. The long-term fungicidal effect against the yeast suspensions was confirmed after 7 days of incubation at 37 °C. For all strains tested, the association of 25 mg l^{-1} with 37.5 J cm^{-2} resulted not only in the absence of colonies on plates but also in the negative growth in the TSB tubes, which indicated that no revival occurred after PDT. In addition, exposure to LED light alone or Photogem® alone had no effect on the viability of all strains evaluated. No significant variations in CFU ml^{-1} among P+L−, P−L+ and P−L− conditions were observed.
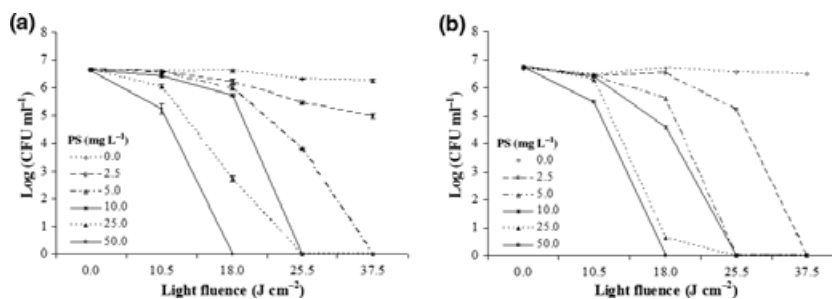

Figure 2 Graphic representation of the effect of PS concentration and light fluence on *Candida albicans* viability. (a) Mean values and standard deviation from logarithmic of survival counts (CFU ml^{-1}) of the three fluconazole-resistant *C. albicans*; (b) values from logarithmic of survival counts (CFU ml^{-1}) of ATCC *C. albicans*.

Figure 3 Graphic representation of the effect of PS concentration and light fluence on *Candida glabrata* viability. (a) Mean values and standard deviation from logarithmic of survival counts (CFU ml^{-1}) of the three fluconazole-resistant *C. glabrata*; (b) values from logarithmic of survival counts (CFU ml^{-1}) of ATCC *C. glabrata*.
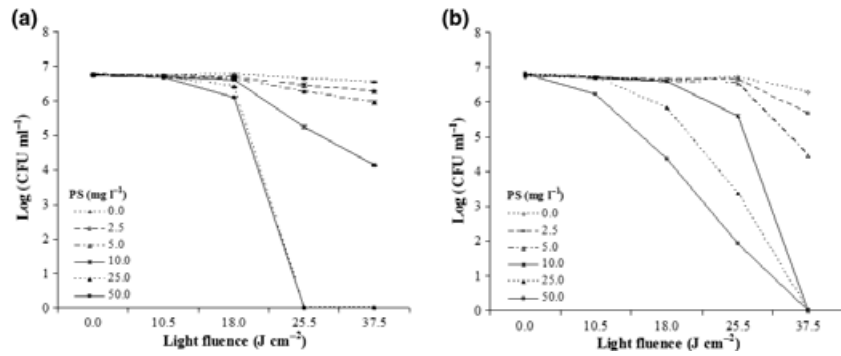
Table 2 Minimal Photogem® concentration (mg l^{-1}) for the photoinactivation of all *Candida* strains.

| | Minimal lethal concentration (mg l^{-1}) | | | | | | | |
| | Candida albicans | | | | C. glabrata | | | |
Light fluence (J cm^{-2})	ATCC	10R	15R	23R	ATCC	50R	63R	87R
10.5	_^{1}	_^{1}	_^{1}	_^{1}	_^{1}	_^{1}	_^{1}	_^{1}
18	50	50	10	10	_^{1}	_^{1}	50	_^{1}
25.5	5	5	10	10	_^{1}	25	25	25
37.5	2.5	5	5	5	10	25	25	25

^{1}Complete inactivation was not observed.

It can be seen from Fig. 4 that PDT reduced the viability of *C. albicans* and *C. glabrata* biofilms when membranes were exposed to 25 mg l^{-1} of PS and illuminated at 37.5 J cm^{-2} (P+L+). Significant decreases in viable counts were observed for *C. albicans* ATCC, 10R and 23R ($P < 0.05$), and for *C. glabrata* ATCC and 50R ($P < 0.05$). Nevertheless, the reduction in viable counts was smaller than 1 log$_{10}$-unit in all experiments. Positive control biofilms showed substantial microbial growth on plates after incubation. There were no significant differences in the log$_{10}$-unit among the biofilms generated with the reference strains and clinical isolates belonging to the same species. However,

the mean number of log$_{10}$-unit for *C. glabrata* was significantly higher than those observed for *C. albicans*.

Discussion

One of the most promising aspects of PDT is that organisms resistant to conventional antifungal agents could be killed by the oxidising species generated by light excitation.[20,23,32] In the present study, PDT was effective in inactivating planktonic suspensions of the three FR clinical isolates of *C. albicans* and *C. glabrata*, using Photogem® with LED light. This finding agreed with published studies in which substantial killing of azole-resistant strains of *C. albicans*[17,23,32] and *C. glabrata*[32] was achieved with the use of toluidine blue,[17] methylene blue[23] and cationic porphyrin[32] as PS. An important observation, however, was that FR strains had different responses to PDT from those of the ATCC strains. At 37.5 J cm^{-2}, higher concentrations of Photogem® were required to achieve the photoinactivation of the FR *Candida* species in comparison with the ATCC strains. Similar results were reported by Jackson *et al.* [17], who observed that PDT was less efficient in killing azole-resistant strains of *C. albicans*. Reduced sensitivity of resistant pathogens to PDT seems to occur not only for *Candida* species, but also for some bacterial strains, such as methicillin-resistant strains of

Figure 4 Graphic representation of the mean values and standard deviation from logarithmic of survival counts (CFU ml^{-1}) of *Candida albicans* (a) and *Candida glabrata* (b) biofilms. P+L+ represents the biofilms treated with 25 mg l^{-1} of Photogem® and irradiated at 37.5 J cm^{-2}; P−L− represents control biofilms. Asterisks (*) represents significant differences from control (P−L−).
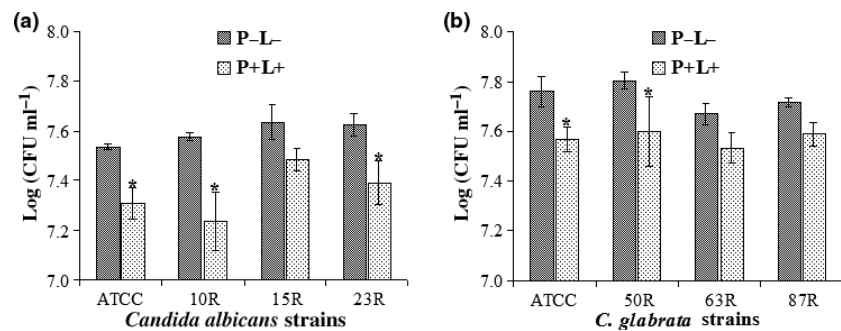
Staphylococcus aureus.[29,33] Thus, it is possible that mechanisms of resistance to traditional drugs can interfere in PDT effectiveness. Some investigations have suggested that azole resistance can be associated with point mutations and overexpression of the Erg11 gene, resulting in alterations in membrane lipid fluidity.[14] It was also reported that *in vitro* deletion of the Erg11 gene of FR *C. glabrata* strains allowed the organisms to become extremely susceptible to oxidative killing.[34] As PDT mediated by porphyrins initially target the plasma membrane, alterations in this organelle can possibly influence the effectiveness of PDT. Therefore, it can be suggested that the modifications associated with Erg11 overexpression might affect the sensitivity to PDT. However, further investigations should be conducted to test the effectiveness of PDT against isogenic mutants in which the Erg11 has been disrupted.

Because the association of 25.0 mg l^{-1} of Photogem$^\circledR$ with 37.5 J cm^{-2} of blue LED light (50 min of illumination) resulted in inactivation of all cell suspensions, these parameters were selected to be tested against single-species biofilms. The *in vitro* model adopted for biofilm formation was based on the methodology described by Spratt *et al.* [35], and it has been successfully applied in several investigations on antimicrobial agents.[35–37] Control membranes contaminated with *C. albicans* and *C. glabrata* strains produced substantial microbial growth on plates after 4 days of incubation. The mean colony count (log$_{10}$ CFU ml^{-1}) for *C. glabrata* biofilms was significantly higher than that observed for *C. albicans*. There have been frequent reports of the superior adherence of *C. glabrata* to different surfaces[38,39] and this can be attributed to its smaller size and higher hydrophobicity, compared with *C. albicans*.[38] When biofilms were exposed to 25.0 mg l^{-1} of Photogem$^\circledR$ with 37.5 J cm^{-2} of blue LED light (50 min of illumination), significant reduction in cell viability was observed. However, the reduction rates were less than 1 log (Table 3), suggesting that the organisms grown on biofilms may have reduced sensitivity to photodynamic damage. This finding may be explained due to structural differences between planktonic and biofilm-grown cells.[18,40,41] Donnelly *et al.* [22] recommended longer application times of PS to obtain PDT response when biofilms were implicated. In addition, Chabrier-Roselló *et al.* [32] reported the use of higher Photofrin$^\circledR$ (Wyeth-Ayerst Lederle Inc., Dublin, Ireland) concentrations to compensate the increased organism biomass of *C. albicans* biofilms in comparison with germ tubes. Accordingly, high concentrations of methylene blue (450 and 500 mg l^{-1}) were used in association with 275 J cm^{-1} to eradicate completely *C. albicans*

Table 3 Log reduction rates obtained after PDT (25 mg l^{-1} of Photogem and 37.5 J cm^{-2} of light) against planktonic and biofilm cultures.

	Culture	
Strains	Planktonic	Biofilm
10R	6.65	0.34
15R	6.66	0.15
23R	6.63	0.23
ATCC 90028	6.66	0.23
50R	6.76	0.20
63R	6.72	0.14
87R	6.75	0.13
ATCC 2001	6.72	0.19

from induced infection of mouse tongues.[23] Nevertheless, the occurrence of cytotoxic effects on healthy cells should be considered before clinical application of such high PS concentrations. The results of the present study showed no dark toxicity of Photogem$^\circledR$ against *C. albicans* and *C. glabrata* cells. In addition, *in vivo* investigations have observed no adverse effects of the dyes, toluidine blue and methylene blue, on periodontal[42] and mucosal[23] structures, in spite of using high dye concentrations. On the other hand, substantial damage to fibroblasts has been reported when high concentrations of a cationic porphyrin were used.[43] Thus, further *in vivo* investigations are necessary to evaluate the cytotoxic potential of haematoporphyrin derivatives before clinical applications of antimicrobial PDT. Animal models of oral candidiasis could be an alternative to verify whether the surrounding tissue and normal microbial flora will not be damaged after PDT mediated by high porphyin concentrations.

Finally, it is important to emphasise that investigations concerning the use of PDT against *Candida* are usually performed with the use of one reference strain[21,44–46] or one clinical isolate.[23] We evaluated the effect of PDT against four *C. albicans* and four *C. glabrata* strains and observed that response of strains to PDT was not homogenous among the three FR strains of the same species. This result agreed with those of Lambrechts *et al.* [47] who reported some significant variation in the sensitivity to PDT of three wild-type strains of *C. albicans*. In the present investigation, single-species biofilms of each FR microorganism showed different responses to PDT. The variation among FR strains of the same species in the manner in which they responded to PDT may have important clinical relevance. It indicates the importance of investigating more than one isolate belonging to a single species, before drawing conclusions with regard to the inter-species differences in susceptibility to PDT. The present inves-

tigation also found that *C. glabrata* strains were less susceptible to PDT when compared with *C. albicans*. In agreement, Bliss *et al.* [20] performed PDT with Photo-frin® concentrations ranging from 10 to 0.01 mg l⁻¹ and illumination with Hg arc lamp against *C. glabrata* and *C. albicans*. The authors noticed that the former species showed less sensitivity to photoinactivation. One possible explanation is the phenomenon of co-adhesion between closely apposed blastoconidia reported by Luo and Samaranayake [38], which may limit the contact area between the *C. glabrata* cells and the PS.

Although some studies have shown the fungicidal effect of laser light without a photosensitiser,[48,49] the results of the present investigations disagree with these findings, because LED light alone does not result in toxicity to yeast cells. In addition, the use of Photogem® alone did not induce toxicity in the strains evaluated. Thus, the killing rates observed in this study were due to the photodynamic effect. In conclusion, the results of this investigation demonstrated that FR *C. albicans* and *C. glabrata* may present reduced sensitivity to PDT, in comparison with reference strains. Microorganisms organised in biofilms appeared to be less susceptible to photoinactivation when compared with planktonic cells. Furthermore, it seems that there may be an inter-strain variation in the susceptibility to PDT. Considering the results presented, *in vitro* investigations of PDT against FR strains are indispensable steps before conducting *in vivo* evaluations. However, *in vivo* conditions were not simulated in this investigation and the parameters used may be not adequate in a clinical situation.

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